THE DEVELOPMENT OF PIROPLASMA CANIS IN CULTURE.

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Plate XIX and 1 Text Figure.

In the course of their investigations upon different species of *Piroplasma* numerous observers have kept infected blood in the ice-chest, or at room temperature, for various periods of time prior to the inoculation of animals. On the evidence afforded by microscopic examination alone some observers have concluded that the parasites are capable of considerable multiplication in defibrinated blood kept *in vitro*. Under these conditions many corpuscles undergo haemolysis and in consequence the parasites appear to be more numerous. We believe that this source of error explains the conclusions arrived at by Lignières and others with regard to the multiplication of *P. bovis* in extravascular blood (see p. 253).

It is only within the last few years that systematic attempts have been made to *cultivate* the different species of *Piroplasma*. We have previously referred (1905, *Journ. of Hygiene*, v. 245) to the negative results which followed the attempts of Nocard and Motas (1902, pp. 274—275) to cultivate *P. canis*, although they established the fact that the parasites remained alive and virulent in blood which had been preserved in the dark and cold for 25 days. Kinoshita (1907, p. 111) has kept the parasite alive for 31 days on ice. In an earlier paper we have described the forms of parasites observed in defibrinated dog's blood up to 48 hours after its removal from the animal.

Kleine's observations.

Kleine (1906, pp. 10—15) states that most of his attempts to cultivate *P. canis in vitro* proved negative until he adopted Robert Koch's suggestion and studied the early changes which take place in cultures made by diluting defibrinated piroplasma blood with salt solution. Kleine infected young dogs by the intraperitoneal injection of about

10 c.c of defibrinated piroplasma blood. When the parasites became numerous in the blood of the infected dogs (they usually died on the third or fourth day) he bled the animals to death under chloroform, defibrinated their blood and added 0·5 c.c. of blood to 0·5 c.c. of salt solution. A series of 20 such cultures in test tubes were made at one time and kept at 27° C.

After the lapse of 18 hours Kleine decanted the clear supernatant fluid from the tubes and upon examining the deposit found that it contained bodies corresponding in form to those described by Koch (1905, 1906) in piroplasma infected ticks. Many of the parasites occurred in the form of clubs with about six rays protruding from the broader portion, whilst the tapering extremity also bore two or more radial processes. The parasites appeared peculiarly rigid, but, on closer examination in drop culture, amoeboid movements could be detected, the parasites becoming slowly rounded or elongated, whilst the length and number of the radial processes changed. These changes were already observable after eight hours, but were most marked after 18 hours.

After 48 hours some parasites had attained a length of 14 μ and a width of 4 μ, the rays at the broad end measuring 9 μ, those at the opposite end measuring up to 19 μ in length. These large parasites occurred together with parasites of normal size. When stained by Giemsa's method the large forms with radii showed a large chromatin mass at the end of the club, and usually a secondary chromatin mass at the tapering extremity. Kleine states that the rays appear blue, unless intensely stained when they take on a red colour. When the parasites were numerous in the blood, masses of radiate bodies occurred, the tapering extremities of the parasites converging to a common centre. When the parasites were very numerous the development of these large forms appeared to be inhibited. At times Kleine observed "fused forms" such as Koch has described in ticks, sausage shaped bodies with radii protruding from the extremities and containing large masses of chromatin at the ends of the sausage and secondary masses of chromatin situated about midway along their length.

After two days at 27° C. the parasites appeared on the whole larger than normal. In forms from early cultures both chromatin masses stain in the same way, but in those derived from cultures two days old the chromatin mass within the clubs retains the peculiar black-red appearance, whilst the larger chromatin mass surrounded by radii appears more lightly stained. After 2—3 days the parasites cease to

exhibit radii, they become rounded and an achromatic zone appears round the dark chromatin mass. The number of degenerating forms increases after the third day. The process of multiplication in defibrinated and diluted blood could not be observed, and no multiplication took place when the developmental forms were transferred to fresh normal or diluted blood.

Kleine thinks that in canine piroplasmosis the parasite usually lies upon the red blood corpuscle. We have elsewhere stated our reasons (*Journ. Hygiene*, VI. 636; VII. 250) for regarding these parasites as being intracorpuscular, and see no reason for changing our opinion[1]. Kleine also considers that the cultivation forms with radii are usually epicorpuscular, but here again we hold a contrary opinion. Owing to its extreme delicacy and lack of staining power the corpuscular envelope may perhaps have escaped his notice in many instances. Occasionally the corpuscular membrane cannot be demonstrated and then the parasite appears to be free.

The writers' experiments.

In our attempts to cultivate *P. canis* under artificial conditions we have made use of several different methods and media, which are described in the following pages. Under most of these conditions the parasites rapidly degenerated without exhibiting any signs of multiplication, or changes of form suggesting further development. These negative experiments (A—L) are first described.

In the last series of experiments (M) however certain large forms with long radiating processes were encountered, similar to those described by Kleine and Koch.

A. In order to ascertain whether the parasites would enter and multiply in normal dogs' corpuscles kept outside the body under artificial conditions the following experiments were made. Blood-stained *serum* taken shortly after death from the heart of a dog dead of piroplasmosis was centrifugalised, and drawn up into capillary pipettes.

[1] Kleine's statement and that of Kinoshita (1907, p. 309) regarding the epicorpuscular position of *Piroplasma canis* appears to be based upon deceptive appearances in stained preparations. Kleine's paper is illustrated by excellent coloured figures and microphotographs which show appearances essentially similar to those we have observed. We have not however seen large agglomerations of radiate bodies such as he figures on Plate IV, fig. 14, and Plate V, fig. 12.

Kinoshita apparently confined his attention to the study of stained preparations of *P. canis*. He has consequently advanced various hypotheses regarding the development of the parasite which we do not consider to be justified.

At the end of an hour the clear fluid contained some free motile pyriform bodies. Some of this fluid was now added to defibrinated normal dog's blood and unstained preparations of the mixture thus obtained were examined. Although free swimming parasites were kept under observation, with one exception which we have previously described (*Journ. of Hygiene*, 1906, Vol. VI. p. 632), none were seen to enter fresh corpuscles.

In other experiments some of this fluid was mixed with normal dog's corpuscles suspended in sodium citrate solution, and the mixture kept in the dark at room temperature for 20 hours. At the end of this time fresh preparations were examined and motile parasites seen, but none were found in corpuscles. Stained specimens confirmed the observations made on living preparations.

Similar experiments were carried out with serum obtained from blood which was allowed to clot, with the same results.

B. Dog's serum containing motile pyriform bodies was added in small quantities to tubes containing rabbits' blood corpuscles suspended in 4 % sodium citrate solution. Examinations of fresh and stained preparations showed that the parasites had not entered the corpuscles.

C. The following experiments were undertaken to determine whether the parasites would live and multiply in the serum of infected animals. Shortly after death blood-stained serum from the heart was drawn up into tubes and centrifugalised. Two hours later the clear fluid showed many rounded parasites and a few actively motile pyriform bodies. After 17 hours a few rounded forms were found, but no motile parasites.

Serum taken from coagulated blood was examined in the same way. This also showed some rounded forms and a few motile pyriform bodies after two hours, but no motile forms after 22 hours.

D. Several experiments were made with *defibrinated blood* taken from the heart immediately after death. Some samples were kept in sealed tubes, and others in tubes plugged with cotton wool. Some of these tubes were kept in the ice chest, and others at room temperature. Examinations of fresh and stained preparations were made at various times with samples taken from the various layers into which the fluid separated. In all 15 experiments were carried out and the examinations were made at times varying between 17 hours and 69 days. Motile pyriform bodies were noticed up to 17 hours, but not later, and non-motile pyriform bodies were seen up to the third day. After this time all the free parasites seen were either rounded or in various stages

of degeneration. The majority of the intracorpuscular forms soon became rounded, but a few of the pyriform ones retained their shape for many days. Occasionally large numbers of rounded parasites were found within leucocytes. (See Plate XIII, fig. 3, *Journ. of Hygiene*, Vol. VI. 1906.)

In some cases the red blood corpuscles retained their normal shape up to 35 days.

E. Eleven experiments were made with blood agar in the hope of obtaining cultivation forms. The blood agar was made by mixing equal parts of defibrinated rabbit's blood with melted agar at 55° C. and was prepared one hour before use. Small quantities of fresh defibrinated heart's blood of dogs dead of the disease were placed in the tubes, which were cultivated at various temperatures. Examinations made at various times revealed only a few degenerated parasites.

F. Defibrinated dog's blood added to various quantities of 2% sodium citrate solution was kept under the same conditions and examined in the same way as in experiment D. Seven experiments were made, the times of the examinations varying between 28 hours and 72 days. Even after 28 hours the majority of free forms and intracorpuscular parasites had become rounded. After this time very few free parasites were seen. In one experiment a few forms with well marked slender radiating processes like those described later (p. 252) were seen. In some cases the red blood corpuscles were found well preserved up to 53 days.

G. Five similar experiments were made by adding defibrinated blood to slightly acid 2% sodium citrate solution with the same results.

H. In three experiments defibrinated blood was added to 4% sodium citrate solution and the cultures examined at various times between 13 hours and 23 days. With very few exceptions both the intracorpuscular and free parasites were found to be rounded. Many of the red blood corpuscles retained their shape up to the 23rd day.

I. Two experiments with normal saline solution containing 4% sodium citrate gave similar results.

J. In six experiments defibrinated blood was added to 25% potassium oxalate solution and specimens were examined between 36 hours and 62 days. The same changes were noted as in experiment F. In some cases the red blood corpuscles retained their normal shapes up to 60 days.

K. Six experiments with slightly acid potassium oxalate solution gave the same results.

L. Following the method of Miyajima (1907) (see p. 255) numerous cultures were made by mixing defibrinated blood and ordinary nutrient broth in proportions varying between 1—5 and 1—10. These were kept for various periods at temperatures ranging between 18° C. and 35° C. Though the red blood corpuscles were well preserved the parasites rapidly became rounded and lost their motility. No developmental forms like those described by Miyajima were seen and none with radiating processes.

M. In the series of experiments about to be described certain very large irregular intracorpuscular forms with radiating spike-like processes were occasionally met with, apparently identical with the cultural forms described by Kleine, and resembling the free parasites observed by Koch in the early stage of infection in the tick.

In the following description these are spoken of as forms with radiating processes and the processes as radii.

In all the following experiments the cultures were made by adding a quantity (about 0·5 c.c.) of blood, defibrinated by shaking with glass beads for 20 minutes, to an equal quantity of 0·6% or 0·8% salt solution or to a physiological saline solution (hereinafter referred to as "P" solution) with the following composition:

Sodium chloride	0·95%
Potassium chloride	0·025%
Calcium chloride	0·02%
Sodium hyd. carbonate ...	0·15%
Dextrose	0·1%
Distilled water	100 c.c.

Dog I. The cultures were made in 0·6% salt solution. Some of these cultures were kept in test tubes plugged with cotton wool, and others in Petri dishes, slightly tilted, at various temperatures (14° C., 16° C., 20° C. and 32° C.). Examinations of fresh and stained preparations were made at various times. At 14° C. after 20 hours a few forms showed short blunt processes. At 16° C. no forms with radiating processes were seen. At 20° C. after 16 hours one form with short radii was found, but at 32° C. several parasites showing radii were observed after 24 hours. These experiments seemed to indicate that a temperature between 20° C. and 32° C. was the most suitable for the development of forms with radiating processes, and consequently in the subsequent experiments the cultures were kept at 24° C.—26° C.

Dog II. Cultures were made in 0·6% salt solution and were kept

at 26° C. After 20 hours a few forms such as are depicted in Plate XIX, figs. 2—4, were seen in corpuscles which had lost their haemoglobin, but none with true radii were found.

Dog III. Several cultures were prepared in 0·8% salt solution and examined at various times, but no forms of interest were met with.

Dog IV. Cultures were made in 0·6% and 0·8% salt solution and kept at 24° C. for 27 hours. Several preparations made from the surfaces of the corpuscular layers of various cultures all showed numerous parasites with radiating processes from which the specimens shown in Plate XIX were drawn.

Preparations made from the other layers and the supernatant fluid showed very few if any parasites with radii.

Dog V. Ten cultures were made in 0·6% salt solution and kept at 24° C. Examinations at 17 and 25 hours were all negative as regards parasites with radiating processes. After 41 hours a single form with true radii was found.

Dog VI. A very complete series of cultures and examinations were made in this case. Cultures in 0·6% salt solution were kept at 24° C. Preparations made after 15 hours and after 25 hours showed no parasites with radii. Examinations after 43 hours, however, showed numerous large forms and some with true radii. These forms were still present a few hours later.

No forms of interest were noted in cultures made with 0·8% salt solution after 25 hours. After 43 hours several large forms were seen and one with true radii. Five hours later these forms were more numerous.

In "P" solution no interesting forms were seen after 15 and 24 hours. After 43 hours three forms with radiating processes were noted, and a few after 48 hours.

Dog VII. In this case all cultures were kept at 24° C. and were left absolutely undisturbed until they were examined.

In 0·6% salt solution after 28 hours parasites were numerous and a few forms with radii were seen. After 27 hours' cultivation no obvious changes were noticed, but after 73 hours many forms with well marked radiating processes were seen.

In 0·8% salt solution a few forms with radii were seen after 28 hours, and after 47 hours they were numerous, but after 73 hours their numbers had decreased.

In "P" solution no forms with radii were seen after 28, 47 and 73 hours' cultivation.

Dog VIII. In this case all the cultures were kept at 24° C. In 0·6 % salt solution after 18 hours large forms were numerous, some of which had radiating processes.

Examinations made after 32 and 48 hours' cultivation showed many large forms, a few of which possessed well marked radii.

In 0·8 % salt solution and in "P" solution large forms were seen, but none with true radii.

Dog IX. Cultures were made in 0·8 % salt solution and were kept at 24° C. After 30 hours many large pyriform bodies were seen, a few of which showed radiating processes, but after 44 hours both the large forms and those with radii were less numerous. Similar cultures in 0·6 % salt solution and in "P" solution showed no forms with radiating processes.

Dogs X, XI, XII, and *XIII.* Similar experiments were made with blood derived from these animals, but no forms with radii were seen.

Dog XIV. Cultures made in 0·8 % salt solution and kept at 24° C. for 26 hours showed numerous forms with well marked radii. Motility was observed in some of them.

Dogs XV, XVI, XVII, XVIII, and *XIX.* Similar experiments were made with the blood of these animals but no forms with true radii were seen.

From the foregoing account it can be seen that in some experiments numerous forms with radiating processes were encountered, whilst in others, apparently conducted under exactly similar conditions, none were found. We have been entirely unable to find any cause for these differences in the behaviour of the cultures.

The differences apparently do not depend on the age of the animal from which the blood was taken, the height of the fever, or the rapidity with which it developed. Nor do they appear to depend on whether the temperature was falling or stationary, or on the period at which the blood was taken either in regard to the date of inoculation, or to the time at which the fever appeared. The following table summarises the data on which these statements are based.

The forms with radiating processes were found most commonly in cultures made in 0·6 % salt solution, and were most frequently obtained from the uppermost part of the layer of blood corpuscles. Although it appears to be necessary to use small quantities of fluid, probably in order to ensure the presence of a sufficient oxygen supply, we did not find that better results were obtained in shallow tilted dishes, with extremely shallow layers of fluid. Cultures which were left absolutely

at rest gave better results than those which were occasionally or continuously shaken, or through which a current of air was intermittently passed. The times at which the forms with radiating processes were most numerous varied in the successful cultures. In dog VIII they were numerous after 17 hours' cultivation, and were fewer after 44 hours. In most cases however they were most numerous on the second day. After that time they usually decreased in numbers.

Positive experiments.

Dog	Age	Temperature: At death	Temperature: Mode of rise	Condition when blood taken	Date after inoculation	Days after fever commenced
I.	puppy	103 °F.	—	stationary	3 days	2 days
IV.	,,	103·8	slow	falling	9 ,,	4 ,,
V.	,,	105·8	,,	stationary	7 ,,	5 ,,
VI.	,,	106	,,	,,	6 ,,	2 ,,
VII.	,,	104	,,	,,	4 ,,	0 ,,
VIII.	,,	103·8	,,	,,	6 ,,	2 ,,
IX.	,,	103·8	rapid	falling	9 ,,	1 ,,
XIV.	,,	103	slow	,,	6 ,,	2 ,,

Negative experiments.

II.	puppy	101·4° F.	slow	falling	12 days	6 days
III.	,,	103	rapid	stationary	10 ,,	2 ,,
X.	,,	—	?	,,	5 ,,	1 ,,
XI.	2 years	104	rapid	,,	4 ,,	2 ,,
XII.	old	103·8	,,	,,	5 ,,	1 ,,
XIII.	puppy	103·6	,,	,,	5 ,,	1 ,,
XV.	,,	103·6	,,	,,	6 ,,	1 ,,
XVI.	,,	102·2	very little	,,	7 ,,	3 ,,
XVII.	,,	104	rapid	,,	4 ,,	1 ,,
XVIII.	,,	103·6	slow	,,	5 ,,	2 ,,
XIX.	,,	—	—	—	—	—

Description of the cultivation forms.

In cultures made in 0·6 % and 0·8 % salt solution forms of the parasite may often be seen, which apparently are not degeneration forms, and which are never met with in the circulating blood or in the organs. The majority of these are large intracorpuscular forms. In very many cases the infected corpuscle has lost its haemoglobin, and its contour can only be made out with difficulty by following the faint line marking the rim of the collapsed and often much enlarged corpuscle. Occasionally no corpuscular remains can be defined and the large parasite appears to be free. Nevertheless we are inclined to think that

most of these apparently free forms are really intracorpuscular, and that failure to find the corpuscular envelope is due to its extreme delicacy and lack of staining power.

The least differentiated forms are large definitely intracorpuscular parasites, such as are figured in Plate XIX, figs. 2—4. Some of these show two well defined masses of chromatin (fig. 1) while others, usually of irregular shape, show three or more masses (figs. 2, 3, 4). Very rarely extremely large forms are seen with several masses of chromatin apparently connected together by thin strands of chromatin (fig. 21). Other examples of intracorpuscular parasites of the same general type show minute, delicate, radiating processes (previously described as radii) projecting beyond the parasites, and usually originating in the neighbourhood of a chromatin mass (figs. 5, 6). Others show much longer processes, frequently long enough to reach the margin of the corpuscle (figs. 7, 8, 9). These processes, which are often extremely delicate, especially at their distal extremities, vary greatly in number, sometimes being almost too numerous to count (fig. 12).

Occasionally several large parasities of this type are seen in one corpuscle (fig. 20).

In yet more remarkable forms some of the processes cause small projections or even considerable distortions of the corpuscular envelope (figs. 9, 11, 13), and occasionally may even perforate the envelope and project for a considerable distance beyond it (figs. 13—16). Similar forms (figs, 17, 18, 19), apparently free, are occasionally seen.

In living preparations all intracorpuscular parasites, except some of the rounded forms, show slight movements, probably of a molecular nature, since small masses of detritus with active dancing movements are often seen within the envelopes of the collapsed corpuscles.

On several occasions we have kept under observation living examples of the forms with long processes and have noticed changes of shape accompanied by the very slow protrusion and retraction of the radiating processes. The accompanying figure illustrates the changes noticed in one parasite (A) during 50 minutes' observation, and in another (B) during a period of five minutes.

Owing to the uncertainty of the cultural methods and the difficulty of keeping living parasites under observation for long periods we have been unable to follow the development of these forms. Possibly the forms with numerous processes develop from forms with many chromatin masses (fig. 21) and the latter by fusion from multiple intracorpuscular forms such as are illustrated in fig. 20.

In the absence of observations on living parasites the interpretation of the appearances seen in stained preparations is extremely hazardous[1] and we do not feel justified in offering any conjectures as to the origin and significance of the bodies we have described.

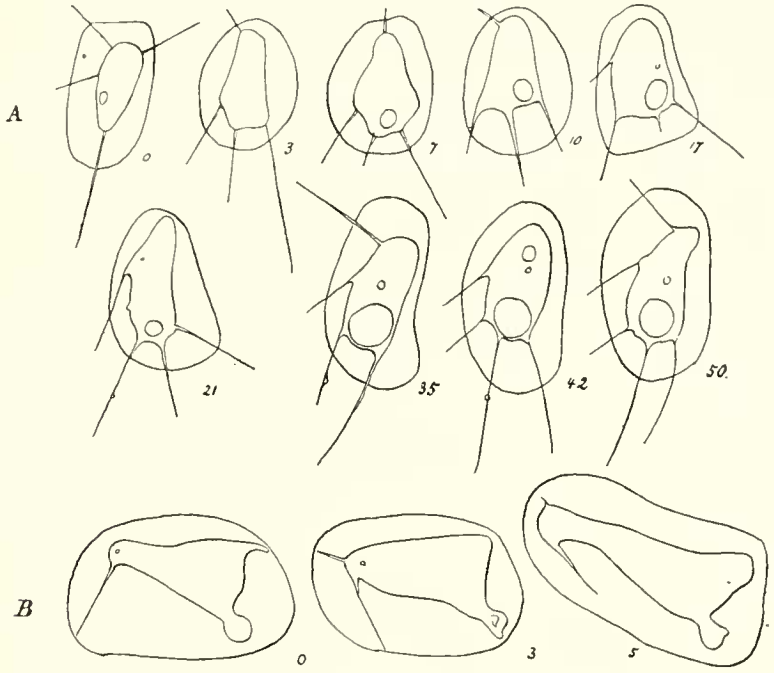

Fig. 1.

Appendix.

1. *Attempts to cultivate the other species of Piroplasma.*

Lignières (cited by Chauvelot, 1904, p. 11) using blood containing many parasites did not succeed in cultivating *P. bovis* in different kinds of broth, gelatin, agar, or on potato, serum, amniotic fluid, or aqueous humor, whether the cultures were exposed to the air, kept in an atmosphere of carbon dioxide, or hydrogen, or kept *in vacuo*. He also obtained negative results with blood kept in collodion sacs. As a culture medium he also tried serum rich in haemoglobin obtained from diseased cattle, adding to it 0·25 c.c. of blood containing many parasites,

[1] Breinl and Hindle (1908) have recently asserted that *P. canis* multiplies in the blood in several different ways. Observations on the forms assumed by the parasite in the living blood during multiplication, which can be studied without great difficulty, do not lend any support to their hypotheses, which are based on the evidence derived from wet films stained by Breinl's method.

which had shown "marked multiplication" at the bottom of tubes containing blood. After 15 days, according to Lignières, the parasites in haemoglobin serum showed marked multiplication and a third subculture gave even better results than did the second. All the culture forms were spherical and he states they contained one to four germs which leave the parent cell, grow rapidly and often remain in pairs connected by a filament which cannot be demonstrated by staining. Lignières (1903, Pl. IV) illustrates such forms in a later publication but his deductions appear to be so largely hypothetical that we do not deem it necessary to consider them further.

Dschunkowsky and Luhs (1904) tried to cultivate the parasites in haemoglobin serum in test tubes. A grayish deposit, which could be shaken up, formed at the bottom of the tubes. They thought they observed evidence of great multiplication at the end of 10—20 days. The parasites also appeared to multiply in defibrinated blood both inside and outside the red blood corpuscles. The parasites were definitely motile and appeared to multiply up to 20—25 days both at room temperature and at 38° C. (It was supposed at the time that they were working with *Theileria parva.*)

Piroplasma ovis.

Motas (1904, p. 31) attempted to cultivate this parasite in blood, blood serum and haemoglobin serum at different temperatures. His results were all negative. He notes that the blood gradually loses its virulence when preserved at 15—18° C., but may remain virulent up to 10—14 days. The blood remains virulent for longer periods, up to 15—20 days, if maintained at lower temperatures, provided no bacterial growth takes place. If the tubes become contaminated virulence is only retained up to 5—10 days. The parasites did not live nearly so long when citrate or oxalate of potash were added to the blood. The virulence was not affected by an exposure of 12 hours to minus 5—6° C. or by exposure to 43—44° C. for the same time.

Piroplasma equi.

Theiler (1903, p. 99) states that he has on several occasions kept the blood of horses containing this parasite *in vitro.* The blood was placed in test tubes and kept at different temperatures, in the ice box, at room temperature, and in the incubator. In the incubator the parasites disappeared from the blood as soon as the corpuscles lost their

haemoglobin. When the blood was kept at room temperature they persisted longer. In blood kept in the ice box the parasites after 12 days appeared as distinct as in fresh blood, but in most cases had become spherical, and were situated at the edges of the infected blood corpuscles. Very exceptionally free parasites were encountered. Some of the corpuscles which contained parasites did not stain in the vicinity of the parasite, so that the latter appeared surrounded by a colourless zone. Theiler suggested that this zone might be due to the destruction of the haemoglobin by some excretion product of the parasite. Although he transplanted the parasites into fresh serum he was unable to observe any multiplication such as Lignières states he observed in the case of *P. bovis*. He regarded the parasites which stained well after 12 days *in vitro* as alive.

Bowhill (1905, p. 2) mixed infected horse blood with potassium citrate in a flask and kept it at room temperature. After 24 hours many circular and oval extra-corpuscular parasites arranged in irregular masses were seen. Stained by the Romanowsky method the chromatin and protoplasm stained red and blue respectively. On adding fresh serum to infected blood and keeping the latter at room temperature, at 29° C. or at 40° C. he was unable to detect any multiplication of the parasites, although on one or two occasions he observed amoeboid movement.

Theileria parva (= *Piroplasma parvum*).

We have elsewhere (IX., 1908, p. 516) stated our reasons for excluding this parasite from the genus *Piroplasma*, but since it belongs to an allied genus it is desirable to consider the cultivation experiments of Miyajima (1907, p. 84).

This observer, working in Japan, states that he tried to cultivate the parasite in blood agar, sodium citrate (acid and alkaline), beef extract, peptone water, calf-serum, normal saline solution, and common broth, to which media he added infected blood. He obtained positive results when he added *Theileria* blood to ordinary broth in the proportion of 1—5 to 1—10 and maintained the cultures at 20—30° C.[1]

"The development of the parasites in a successful culture takes place in the following manner: on the first day no motile form is seen;

[1] Miyajima states incidentally that he was able to cultivate *Tr. lewisi* under similar conditions. Although we have tried to cultivate this parasite on several occasions according to Miyajima's method we have not succeeded in doing so.

on the second, there can be observed a certain number of peculiar cells which occupy the upper layer of the sedimented corpuscles and which microscopically appear as a series of white dots. Very few motile forms resembling trypanosomata are visible in these cells on the third day of incubation, but thereafter the trypanosomata multiply vigorously and reach the maximum number between the tenth and fourteenth day."

"In a culture kept at room temperature, the trypanosomata remain motile until 45 days later, at this time most of them have undergone degeneration and globular cells with irregular granulation result."

When kept at 10—20° C. the trypanosomes were alive after a lapse of three months.

Miyajima states that he was able to maintain the parasites alive in subcultures as Novy and others had done with *Tr. lewisi.*

According to Miyajima *Theileria parva* therefore appears to develop into a trypanosome under cultural conditions in blood added to broth. Nine of the 21 native cattle examined showed *Theileria parva* and from the blood of seven out of the nine trypanosomes were obtained in cultures. The transplantation of a single loopful of blood was sufficient to secure a positive culture. The development described by Miyajima appears to be extremely rapid. After three days at 25° C. the diminutive *Theileria* is stated to have attained or exceeded the size of a red blood corpuscle, and to have become actively amoeboid. Very large vacuolated cells appear after 20 hours and curious crescentic bodies after 48 hours which give rise to typical flagellates. After 72 hours besides the nucleus and blepharoplast the flagellates exhibit an undulating membrane and can be seen to divide longitudinally, gradually giving rise to large colonies.

As controls Miyajima examined 200 cattle but *Theileria* were not found in blood films and trypanosomes did not develop in cultures.

Miyajima confirmed the observations which have been made in other countries that *Theileria* cannot be transmitted to clean animals by blood inoculations. On the other hand he found that two out of three clean calves inoculated with cultures containing the trypanosomes became infected in eight days with red-water. One of these animals gave a positive result with cultures (development of trypanosomes) 17 days before the appearance of *Theileria* in its blood upon microscopic examination. If Miyajima's observations are correct they are certainly most interesting and remarkable, but a certain amount of scepticism appears justified until they have been extended and confirmed by other workers. It will be remembered that Schaudinn (1904, p. 438, see

Journ. Hygiene, VI. p. 642) advanced the hypothesis that *P. bovis* undergoes development into a trypanosome, a statement which has gained no support from subsequent investigations. Should *Theileria* develop into a trypanosome we would have an additional ground for separating it, in the light of our present knowledge, from *Piroplasma.*

2. *Blood-platelets and haematozoa.*

In connection with these cultivation experiments Swingle's (1908) observations on the similarity between blood-platelets and certain haematozoa are of interest. He made cultures of normal sheep and other blood in a medium of the following composition: water 1000 c.c., sodium citrate 5 grms., sodium chloride 5 grms. made slightly acid with hydrochloric acid. One drop of fresh blood was added to 2 c.c. of the solution. For making stained preparations he placed a drop of the culture fluid on a slide and after it had evaporated down, but was not entirely dry, he dropped on some killing fluid such as Zenker's solution. The specimens were stained by various methods.

He remarks that "normal platelets resemble normal piroplasma forms in size and general shape. A nucleus, and refractive spots, probably vacuoles, can be seen" (p. 49). We cannot entirely agree with this statement, since in our own preparations we have seldom found any difficulty in distinguishing between blood-platelets and forms of the parasite.

Swingle's observations, which are quoted at length, on the cultivation forms of platelets are extremely interesting. "Cultures examined as quickly as possible after drawing the blood showed amoeboid and flagellate forms. A fact of great importance to the student of haematozoa is that the most of these forms are capable of movement. They seem to roll over, swing round, and often move for a distance equal to the diameter of two or three red corpuscles. Among the amoeboid types there were always present in great numbers platelets with few or several long, sharp, or sometimes blunt, pseudopodia. From the description and figures of Koch and Kleine one must conclude that these forms are very similar to, if not identical with, what they describe as developmental stages of *Piroplasma.* To be sure Koch found his stages in the stomach of the tick, but this does not disprove the statement, for just such forms were also found in the stomach of the sheep tick after sucking the blood of a sheep....In older cultures most of the platelets that had no pseudopodia were at the rim, either inside or just

outside of a transparent circle about the size or a little larger than the platelet. Those outside looked as if they had crawled out of a thin envelope. As to their significance, I can only say that they apparently were not degeneration forms, inasmuch as they were still active in their amoeboid movements. The more typically flagellate forms, those with a single flagellum, are perhaps of still more importance, because they so perfectly simulate real flagellates. Although they are found immediately after the introduction of the blood into sodium citrate solution, the 'flagellum' is generally quite rigid except at the very end, where it can be seen to vibrate. Notwithstanding this rigidity, they seem to move about, roll over, and swing round, these movements probably being the result of the vibrations at the tip of the flagellum. The most motile forms were found in a culture of human blood kept for the first six hours in an ice chest and after that at room temperature for 50 hours. Round or pear-shaped individuals with a flagellum measuring in some cases as much as 20 μ were found in abundance. In the pear-shaped forms the flagellum is at the pointed end. It was very slender, in most cases appearing and moving very much like the flagellum of *Euglena*, often with lashings violent enough to move the red corpuscles on coming in contact with them. Instead of being smooth, in some instances the flagella had thickened, knotted portions, which bear a close resemblance to Kinoshita's description and drawings of the flagellates which he found. It is important to note that he found the best developed flagellate with a flagellum 15 μ long in sodium citrate culture.

The various forms are often found grouped together in couplets, triplets, or in masses composed of as many as a hundred individuals. In this condition they retain their individual motion, rolling over and turning about. I have seen these masses stained with iron haematoxylin so that they had the exact appearance of Kleine's photograph."

Swingle says that he found such masses in the stomach contents of sheep ticks fed on sheep's blood. "To eliminate the possibility of confusing the platelets with the herpetomonadine flagellates, which are generally present in adult ticks, they were studied in young ticks before the latter had become infected."

"Flagellation of blood-platelets is not limited to sodium citrate culture, but may take place in other media."

"In stained preparations one often finds a most striking, yet doubtless merely coincident, resemblance in nuclear conditions to trypanosome forms. While nuclear dimorphism is not by any means to be

found in all blood-platelets, yet it is by no means rare. And when it does occur there is not such marked distinction between the nuclear masses as one sees in trypanosomes, but still as much distinction as many of Kinoshita's drawings would indicate for *Babesia*....Thus it would be an easy matter to mistake such blood-platelets for real flagellates having true nuclear dimorphism." "The flagella and pseudopodia stain like cytoplasm, and not like chromatin as in the trypanosomes."

Swingle concludes his paper with the following words: "It is not sufficient answer to the similarity I have shown to say that Kleine used defibrinated blood, and hence blood-platelets were not present in his solutions, for no one has demonstrated that the platelets are entirely removed by defibrination. Since blood-platelets in various culture media and in the stomach of the tick always develop flagella, move about, and manifest such a marked resemblance in form, size, and structure to *Babesia* and the Leishman-Donovan bodies, investigators must furnish criteria to differentiate between the flagellated platelets and the parasites. Until they have established their position by experiments with normal blood, the correctness of their results can be accepted only with some reserve. The evidence I have presented shows that neither are motion and flagellation exclusive characters of parasites nor will they differentiate them from blood-platelets. Each student will have to determine experimentally how to distinguish the two classes of structures."

As controls for our observations we have carried out a number of experiments by Swingle's method, using *defibrinated* blood from normal and infected dogs. Occasionally we have found platelets such as he has described, but the majority of cultures showed none. We do not think that the intracorpuscular forms of piroplasma with radiating processes could be mistaken for platelets, and up to the present we have not met with any "flagellated" platelets which could be mistaken for the extracorpuscular parasites.

DESCRIPTION OF PLATE XIX.

Fig. 1. Large free pyriform parasite with two chromatin masses.
2, 3, 4. Large intracorpuscular parasites each with several chromatin masses.
5, 6. Large intracorpuscular parasites with short radii.
7. Small intracorpuscular parasite with several radii.
8, 9. Intracorpuscular parasites with long radii.
10–16. Intracorpuscular parasites with numerous radii, some of which apparently penetrate the corpuscular envelope.

Figs. 17–19. Large free parasites with long radii.

20. Photograph (× 3000) of corpuscle containing several parasites with short radii.

21. Photograph (× 3000) of corpuscle containing a large triangular parasite with several chromatin masses connected by thin strands.

22, 23. Photographs (× 3000) of corpuscles containing large pyriform parasites with several long radii projecting from their blunt extremities.

24. Photograph (× 3000) of corpuscle containing a large rounded parasite with numerous radii.

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